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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12Q 1/10 // 1/10, C12R 1/42

A1

(11) International Publication Number: WO 96/30543

(43) Internati nal Publication Date: 3 October 1996 (03.10.96)

(21) International Application Number: PCT/FI96/00163

(22) International Filing Date: 20 March 1996 (20.03.96)

(30) Priority Data:
951431 24 March 1995 (24.03.95) FI

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(81) Designated States: AU, CA, CZ, HU, JP, KR, NO, PL, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHOD AND CULTURE MEDIUM FOR IDENTIFICATION OF SALMONELLAE

(57) Abstract

The culture medium and method for distinguishing bacteria of *Salmonella* spp. from other gram-negative bacteria, especially those belonging to the family *Enterobacteriaceae*, is based on the ability of salmonellae to utilize melibiose, mannitol, and sorbitol int acids. This property is made use of together with a chromogenic substrate used for identifying β-galactosidase. Other bacteria of the family *Enterobacteriaceae*, most of which are β-galactosidase-positive, appear as brown, blue, or green colonies, depending on the chromogenic substrate used. Apart from *Salmonella* spp., other β-galactosidase-negative bacteria, such as *Proteus* spp., appear as colorless colonies. Salm nellae can be identified directly on the culture medium after incubation, on the basis of their characteristic bright red color.

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BNSDOCID: <WO___9630543A2_I_>
BNSDOCID: <WO___9630543A1_I_>

METHOD AND CULTURE MEDIUM FOR IDENTIFICATION OF SALMONELLAE

The present invention relates to a method and a culture medium for the identification and distinguishing of *Salmonella* sp. among other gram-negative bacteria, especially those belonging to the family *Enterobacteriaceae*, in an analytical sample. The invention is characterized by the features defined in the claims.

Rapid species identification of infective organisms is important, whether for epidemiology studies, for diagnosis of both human and veterinary diseases, for selecting appropriate medical treatment or for deciding on control measures in the food industry and other segments of environmental hygiene. To prevent outbreaks of food poisoning, food and environmental (water, soil and alike) samples are continuously being monitored especially for the presence of salmonellae.

At least eight different agar-based media for culturing and identification of salmonellae are commercially available today (Difco Manual, 1984, Difco Laboratories, Detroit, Michigan, USA). Most of these are based on determination of lactose utilization and/or measurement of hydrogen sulfide production. Since most salmonellae are lactose-negative and thus do not contain β-galactosidase enzyme as most of the other bacteria in the family *Enterobacteriaceae* they can easily be distinguished from many other bacteria on the basis of their β-galactosidase negativity.

The currently used culture media contain various amounts of additives that inhibit the growth of other bacteria; in other words, the media have been made selective so that only salmonellae would grow on them. These media have many limitations, however. Use of inhibitors that

prevent the growth of gram-negative bacteria is undesirable, since some inhibitors will, to some extent, prevent even the growth of salmonellae. In other words, such media are too selective and therefore two different media have to be used, one of which is less selective. The more selective culture medium is used for measuring the production of hydrogen sulfide, which alone is not a reliable method because it is sensitive to many external factors, such as oxygen concentration and pH. Furthermore, several strains produce different amounts of hydrogen sulfide. Combining measurement of hydrogen sulfide production with lactose fermentation on a less selective medium is also insufficient to distinguish salmonellae from other bacteria occurring in nature. Since i.a. *Proteus* spp. resemble salmonellae in being lactose-negative and in producing hydrogen sulfide, they cannot be distinguished from salmonellae by means of commercial culture media (Difco Manual).

The aforementioned commercial culture media do not allow differentiation among colonies on the basis of appearance, since bacteria form colonies of uniform color on these media. Nevertheless, the recently introduced Rambach agar has been developed with a view to enhancing the distinction of different bacteria directly on the basis of colony color (E. Merck, Darmstadt, Germany). On this agar, *Salmonella* spp. grow as pink colonies while other bacteria of the family *Enterobacteriaceae*, e.g., many coliforms, form blue, green, violet, or colorless colonies. This advantage of Rambach agar is based on the ability of β-galactosidase-negative salmonellae to utilize propylene glycol. In the presence of an indicator substance, decomposition of propylene glycol yields a red color, and not blue, for instance. Rambach agar is very specific for all salmonella strains except *S. typhi* and *S. paratyphi*. Few false positives are obtained with Rambach agar (Garrick R.G. and A.D. Smith, Letters Appl.

Microbiol. 18:187-189, 1994). Still, the method has the disadvantage of not revealing typhi strains.

US patent 5,434,056 discloses a method for selective detection of salmonella, and a medium for that purpose which contains glucuronic acid or its salts, a pH indicator, a cromogenic compound to distinguish β-galactosidase-positive bacteria from β-galactosidase-negative salmonellae, and optionally at least one fermentable sugar. Sugars mentioned include melibiose, sorbitol, dulcitol, mannitol, glucose and glucuronate in concentrations between 1 to 10 g/l. The *Salmonella* sp. are detected as red colonies, the color formation being based on the capacity of salmonella to ferment glucuronic acid or its salts. This method has the disadvantage that *S. arizonae* cannot be distinguished with it. Further, it is stated in the patent that adding sorbitol to the culture medium may reveal bacteria of the *Serratia* genus to be false positives. This leads away from using sugars in the medium, and thus it leads away from the present invention.

Biochemical reactions other than those mentioned above can also be used for rapid identification of salmonellae in biological specimens. These reactions include methods based on the utilization of sugars and sugar alcohols, such as bacterial identification by means of melibiose, mannitol, and sorbitol (Bergey's Manual of Systematic Bacteriology, Vol. 1, p. 408, eds. R.G.E. Murray et al., William & Wilkins, Baltimore, USA, 1984).

We have now developed a medium which contains a pH-indicator, a chromogenic compound to distinguish β-galactosidase-positive bacteria from for example β-galactosidase-negative salmonellae, and a combination of three very carefully selected sugars, i.e. melibiose, mannitol and sorbitol. We have shown that on of these sugars is not sufficient to reliably distinguish all salmonellae as

bright red colonies (see Experimental). All three of these sugars are ne d d to give a reliable result. We have also found that even strains of *Salmonella typhi* can be identified by the new medium now developed, since they utilize mannitol and sorbitol.

Utilization of melibiose, mannitol, and sorbitol in combination with the lack of β-galactosidase activity distinguishes salmonellae from other members of the family *Enterobacteriaceae*. Addition of a pH indicator, such as neutral red, to the agar makes salmonellae stain bright red when producing acid from melibiose and sugar alcohols. The acids lower the pH around the colonies of *Salmonella* spp., and only the salmonellae appear as bright red colonies in the presence of neutral red. Characteristically, other β-galactosidase-negative bacteria of the family *Enterobacteriaceae*, e.g., strains of *Proteus* sp., appear colorless on the culture medium because they do not utilize melibiose or the above-mentioned sugar alcohols. The β-galactosidase-positive bacteria of *Enterobacteriaceae* stain differently from salmonellae. In the method presented by us, they stain brown with the use of the chromogenic 8-hydroxyquinoline-β-D-galactoside, a substrate measuring galactosidase activity.

In order to inhibit the growth of gram-positive bacteria it is preferable to use in the culture medium inhibitory substances such as bile salts or anionic detergents. Sodium dodecyl sulphate and 3,9-diethyl-6-tridecanol sulphate ester are examples of suitable anionic detergents.

An analytical sample which can be assayed using the method according to the present invention can b taken from any organ of th human or animal body. Most frequently salmonella is found in the intestinal canal.

However, the occurrence of salmonella has b en reported also in m ny other organs. Representative body fluids may b , for example, feces, urine, abscess, blood, plasma, serum, liquor, bile fluid, healing wound fluid, ascitic fluid, pleural fluid, synovial fluid, blister fluid, or amniotic fluid. A sample may also be any food, environmental, or industrial specimen.

The method developed by us has the further asset of allowing all ingredients of the culture medium to be premixed to yield a powdered medium, i.e., an instant powder mixture, which only requires addition of water before autoclaving and pouring into Petri dishes. Other corresponding products on the market do not permit pre-mixing of all their components to yield a powdered medium. Rambach agar, for example, consists of a powder and a liquid, propylene glycol. Another asset of the developed method is the stability of the powdered culture medium. The culture medium keeps at least for seven months. If packed well, it also keeps in the form of reconstituted medium, either as plates or dip-slides. The stability of dip-slides is at least six months, when properly stored. Especially the 8-hydroxyquinoline-β-D-galactoside has good stability - unlike other substances, such as the chromogenic indole substrate used in Rambach agar.

The invention will be described in detail with the Examples below. The specific examples are provided as a guide to assist in the practice of the invention, and are not intended as a limitation on the scope thereof.

Experimental

Example 1

The experiments were conducted with bacteria representing clinical strains commonly encountered in diarrheal diseases. The salmonella strains (212 strains) and their identification and typing data were obtained from the Department of Special Bacterial Pathogens, National Public Health Institute, Finland. Of the salmonellae, 100 belonged to the group *Salmonella enterica* ssp. *enterica* (serotypes Enteritidis, Typhimurium, and Infantis), which comprises some of the most common bacterial strains in diarrheal diseases. The remaining 112 strains belonged to several other serotypes of salmonella. The development of the culture medium and method also made use of the following type strains of the American Type Culture Collection (ATCC): gram-negative bacteria, including *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 27922), *Klebsiella pneumoniae* (ATCC 13883), *Proteus mirabilis* (ATCC 12453), *Pseudomonas aeruginosa* (ATCC 27853), and *Enterobacter aerogenes* (ATCC 13048); gram-positive bacteria, including *Staphylococcus aureus* (ATCC 25922), *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus faecium* (ATCC 9790), β-hemolytic *Streptococcus* sp. of group B, *Enterococcus* sp., and *Corynebacterium* sp.

The bacteria were grown in Brain Heart Infusion broth (BHI, Difco, Detroit, Michigan, USA) at 37 °C for 24 hours. The bacterial concentration was adjusted to 10^8 bacteria/ml by diluting the BHI with sterile 0.9 % NaCl. This suspension was diluted further with 0.9 % NaCl. The dilutions were inoculated onto the solid agar-based culture medium of the present invention and the composition of which is presented in Table 1. The culture medium of the pres nt invention is characterized, i.a., by the

fact that it contains, in addition to established components, mannitol, melibiose, sorbitol, and 8-hydroxyquinoline-β-D-galactoside. The inoculated bact ria w re incubated at 37 °C for 24 to 48 hours. The bacterial count of the inoculants was confirmed by culturing the dilution series on a general culture medium without selective substances, e.g., on nutrient agar; the bacterial count was obtained from the colony count/ml.

Table 1. Composition of the culture medium, pH 7.5-8.0.

Nutrients and other additives	Amount, g/l
Tryptone (Difco)	15.0
Soybean peptone (Oxoid)	5.0
Mannitol (Fluka)	10.0
Melibiose (Fluka)	10.0
Sorbitol (Fluka)	10.0
Agar agar (Oxoid)	22.0
Bile salts (BBL)	1.8
8-hydroxyquinoline-β-D-galactoside (Biosynth)	0.5
Ferric citrate (Merck)	1.0
Neutral red (Merck)	0.02
Distilled water	1000 ml

The culture medium may have a pH of 7.5 to 8.0, preferably 7.8 to 8.0.

Example 2

The ingredients listed in Table 1 were weighed, mixed in distilled water, dissolved by heating, and autoclaved at 121 °C for 15 min, after which the agar mixture was poured into Petri dishes. The bacteria were pre-cultur d in suspension and diluted to a suitable concentration as set out in Example 1.

As seen in Table 2, the gram-negative bacteria grew equally well on the culture medium of the invention as on the nutrient agar. In addition, the table shows that the growth of gram-positive bacteria was inhibited by the bile salts. Only *Salmonella typhimurium* appeared as red colonies, whereas the colonies of the other β-galactosidase-negative species, *Proteus mirabilis* and *Pseudomonas aeruginosa*, were colorless. β-galactosidase-positive coliforms, such as *Escherichia coli* and *Klebsiella pneumoniae*, appeared as brown colonies on the culture medium.

Table 2. Color and bacterial count of colonies of gram-negative and gram-positive bacteria on a cultur medium according to Table 1, compared with nutrient agar.

BACTERIUM	NEW CULTURE MEDIUM bact/ml	Color of colonies	NUTRIENT AGAR bact/ml
Gram-negative bacteria:			
Salmonella typhimurium (ATCC 14028)	10^5	red	10^5
Escherichia coli (ATCC 27922)	10^5	brown	10^5
Klebsiella pneumoniae (ATCC 13883)	10^5	brown	10^5
Proteus mirabilis (ATCC 12453)	10^5	colorless	10^5
Pseudomonas aeruginosa (ATCC 27853)	10^5	colorless	10^5
Enterobacter aerogenes (ATCC 13048)	10^5	brown	10^5
Gram-positive bacteria:			
Staphylococcus aureus (ATCC 25922)	0		10^5
Staphylococcus saprophyticus	0		10^5
Staphylococcus epidermidis	0		10^5
Streptococcus agalactiae	0		10^5
Streptococcus faecium (ATCC 9790)	0		10^5
Streptococcus sp., group B	0		10^5
Enterococcus sp.	0		10^5
Corynebacterium sp.	0		10^5

Example 3

Table 3 shows the growth data and color reactions of gram-negative bacteria on the culture medium of the present invention.

Tabl 3. Numbers of bacterial strains tested and the colors of their colonies on the culture medium.

	Number of strains	Bright red colonies	Brown colonies	Colorless colonies
Salmonella sp.	213	211	2**	0
Other members of the family *Enterobacteriaceae*:				
Escherichia coli	33	0	33	0
Proteus sp.	13	0	0	13
Klebsiella sp.	8	2*	6	0
Enterobacter sp.	3	0	3	0
Citrobacter sp.	2	0	2	0
Serratia sp.	1	0	0	1

* pink colony
** β-galactosidase-positive strain

As can be seen in the table, 211 strains of *Salmonella* sp. formed bright red colonies and two strains of *Klebsiella* sp. formed pink colonies on the culture medium of the invention. Two salmonella strains were β-galactosidase-positive and formed brown colonies, as did other β-galactosidase-positive bacterial strains, including *Escherichia coli*. β-galactosidase-negative bacteria, such as *Proteus* sp. and *Serratia* sp., formed colorless colonies.

Example 4

Comparison was made by cultivating different salmonella strains on media containing one or more of the three substances: mannitol, melibiose and sorbitol.

The results are given in the Table 4. It can be seen that when a combination of mannitol and sorbitol is used only 72% of salmonellae is distinguished as red colonies, whereas a combination of melibiose and sorbitol results in 59% of red salmonellae colonies. When, according to the present invention, a combination of mannitol, sorbitol and melibiose is used, 100% of the cultured salmonellae results in red colonies. The optimal sugar concentrations used are about 10 g/l of each sugar. This is an essential improvement compared to methods of prior art.

Table 4 Performance (colour reaction) of salmonella strains with different sugars on the salmonella medium

Strains tested (n)	Mannitol + Melibiose n (%)	Melibiose + Sorbitol n (%)	Mannitol + Melibiose + Sorbitol n (%)
50			
Bright red colony	36 (72)		50 (100)
Yellow colony	14 (28)		
105			
Bright red colony		62 (59)	105 (100)
Yellow colony		43 (41)	

CLAIMS

1. A method for distinguishing bacteria of *Salmonella* spp. among other bacteria of the family *Enterobacteriaceae*, comprising

- plating an analytical sample on a solid medium containing melibiose, mannitol and sorbitol, a pH indicator, and a chromogenic substrate revealing all β-galactosidase-positive bacteria,
- cultivating the bacteria, and
- detecting the bacteria of *Salmonella* spp. as bright red colonies.

2. A method according to claim 1, wherein the chromogenic substrate is 8-hydroxyquinoline-β-D-galactoside.

3. A method according to claim 1 or 2, wherein the medium further contains inhibitory substances that inhibit the growth of gram-positive bacteria.

4. A method according to claim 3, wherein the inhibitory substances are selected from bile salts and anionic detergents.

5. A method according to claim 1, wherein neutral red is used as the pH indicator.

6. A method according to any one of claims 1 to 5 wherein the analytical sample is a body organ or body fluid sample, or a food, environmental, or industrial specimen.

7. A solid culture medium for distinguishing bacteria of *Salmonella* spp. among other bacteria of the family *Enterobacteriaceae*, comprising melibiose, mannitol and sorbitol, a chromogenic substrate that reveals all β-galactosidase-positive bact ria, a pH indicator, and conventional solidification ag nts and growth factors.

8. A culture medium according to claim 7 wherein the chromogenic substrate is 8-hydroxyquinoline-β-D-galactoside.

9. A culture medium according to claim 7 or 8 wherein the medium further contains inhibitory substances that inhibit the growth of gram-positive bacteria.

10. A culture medium according to claim 9, wherein the inhibitory substances are selected from bile salts and anionic detergents.

11. A culture medium according to claim 7, wherein the pH of the culture medium is 7.5 to 8.0.

12. A culture medium according to any one of claims 7 to 11, wherein the culture medium is in the form of a powdered medium, i.e., an instant powder mixture.

13. A culture medium according to any one of claims 7 to 11, wherein the medium is in the form of a prepared ready-to-use medium, either in Petri dishes or on dipslides.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 96/00163

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/10 // C12Q 1/10, C12R 1/42
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9212259 A1 (BIO MERIEUX), 23 July 1992 (23.07.92) --	1-13
A	WO 9409152 A1 (MERCK PATENT GESELLSCHAFT MIT BESCHRÄNKTER HAFTUNG), 28 April 1994 (28.04.94) --	1-13

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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25 June 1996	09 -07- 1996
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INTERNATIONAL SEARCH REPORT

Information on patent family members

01/04/96

International application No.
PCT/FI 96/00163

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9212259	23/07/92	AT-T- 135051	15/03/96
		DE-D- 69117744	00/00/00
		EP-A,A,B 0516817	09/12/92
		ES-T- 2084345	01/05/96
		FR-A,B- 2671100	03/07/92
		US-A- 5434056	18/07/95
WO-A1- 9409152	28/04/94	NONE	